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(54) Title: METHOD OF SYNTHESIZING SACCHARIDE COMPOSITIONS

(57) Abstract

A method of synthesizing saccharide compositions is described. In this method, an acceptor moiety is contacted with at least one donor saccharide in the presence of at least one cell surface-bound glycosyltransferase specific for catalyzing the coupling of the acceptor moiety with the donor saccharide. The acceptor moiety used is a carbohydrate, a protein, a glycoprotein, a lipid, or a glycolipid.

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Description

Method of Synthesizing Saccharide Compositions

Technical Field

This invention relates to methods of preparing saccharide compositions such as, for example, oligosaccharides, polysaccharides, glycolipids, and glycoproteins.

Background Art

The term "carbohydrate" embraces a wide variety of chemical compounds having the general formula $(CH_2O)_n$, such as monosaccharides, disaccharides, oligosaccharides and polysaccharides. Oligosaccharides are chains composed of saccharide units, which are alternatively known as sugars. These saccharide units can be arranged in any order and the linkage between the two saccharide units can occur in any of approximately 10 different ways. As a result, the number of different possible stereoisomeric oligosaccharide chains is enormous.

Of all the biological polymer families, oligosaccharides and polysaccharides have been the least well studied, due in part to the difficulty of sequencing and synthesizing their often complex sugar chain. Although the synthesis of oligonucleotides and polypeptides are well developed, there is currently no generally applicable synthetic technique for synthesizing oligosaccharides.

Numerous classical techniques for the theoretical synthesis of carbohydrates have been developed, but these techniques suffer the difficulty of requiring selective protection and deprotection, and, to date, have only provided very limited results. Organic synthesis of oligosaccharides is further hampered by the lability of many glycosidic bonds, difficulties in achieving regioselective sugar coupling, and generally low synthetic yield. These difficulties, together with the difficulties of isolating and purifying carbohydrates and of analyzing their structure, has made this area of chemistry a very demanding one.

Intensive research efforts have been devoted to carbohydrates and molecules comprising carbohydrate fragments, such as glycolipids and glycoproteins. Research interest in these moieties has been largely due to the recognition that interaction between proteins and carbohydrates are involved in a wide array of biological recognition events, including fertilization, molecular targeting, intracellular recognition, and viral, bacterial, and fungal pathogenesis. It is now widely appreciated that the oligosaccharide portions of glycoproteins and glycolipids mediate the recognition between cells and cells, between cells and ligands, between cells and extracellular matrix, and between cells and pathogens.

These recognition phenomena can likely be inhibited by oligosaccharides which have the same sugar sequence and stereochemistry found on the active portion of a glycoprotein or glycolipid involved in cell recognition. The oligosaccharides are believed to compete with the glycoproteins and glycolipids for binding sites on the receptor proteins. For example, the disaccharide galactosyl β 1-4 N-acetylglucosamine is believed to be one component of the glycoprotein which interacts with receptors in the plasma membrane of liver cells. To the extent that they compete with potentially harmful moieties for cellular binding sites, oligosaccharides and other saccharide compositions have the potential to open new horizons in pharmacology, diagnosis and therapeutics.

There has been relatively little effort to test oligosaccharides as therapeutic agents for humans or animal diseases however, as methods for the synthesis of oligosaccharides have been unavailable as noted above. Limited types of small oligosaccharides can be custom-synthesized by organic chemical methods, but the cost of such compounds is typically prohibitively high. In addition, it is very difficult to synthesize oligosaccharides stereospecifically and the addition of some sugars, such as sialic acid and fucose, has not been effectively accomplished because of the extreme lability of their bonds. Improved,

generally applicable methods for oligosaccharide synthesis are thereby desired for the production of large amounts of widely varying oligosaccharides for therapeutic purposes.

For certain applications, enzymes have been targeted for use in organic synthesis as one alternative to more traditional techniques. For example, enzymes have been used as catalysts in organic synthesis, where the value of synthetic enzymatic reactions in such areas as reaction rate acceleration and stereoselectivity has been demonstrated. Additionally, techniques are now available for the low cost production of some enzymes and for alteration of their properties.

Glycosyltransferases catalyze the addition of activated sugars (donor NDP-sugars), in a step wise fashion, to a protein, glycoprotein, lipid or glycolipid or to the non-reducing end of a growing oligosaccharide. N-linked glycoproteins are synthesized via a transferase and a lipid-linked oligosaccharide donor [Dol-PP-$NAG_2Glc_3Man_9$] in an <u>en block</u> transfer followed by trimming of the core. In this case the nature of the "core" saccharide is somewhat different from subsequent attachments. A very large number of glycosyltransferases appears to be necessary to synthesize carbohydrates. Each donor NDP-sugar residue requires a distinct class of glycosyltransferases and each of the more-than-100 glycosyltransferases identified to date appears to catalyze the formation of a unique glycosidic linkage. To date, the exact details of the specificity of the glycosyltransferases are not known. It is not clear for example what sequence of carbohydrates is recognized by most of these enzymes.

Glycosyltransferases have been found in soluble form in many vertebrate body fluids, but they are generally in membrane-bound form when associated with cells. Many of the membrane-bound enzymes studied thus far are considered to be intrinsic proteins; that is, they are not released from the membranes by sonication and require detergents for solubilization. Before 1971, glycosyltransferase activities

were generally thought to be localized in the Golgi-retractions and endoplasmic reticulum of cells, since that was the finding in rat liver. Since then, surface glycosyltransferases have been identified on the surfaces of vertebrate and invertebrate cells, and it has also been recognized that these surface transferases maintain catalytic activity under physiological conditions. However, the more recognized function of cell surface glycosyltransferases is for intercellular recognition. (Roth, Molecular Approaches to Supracellular Phenomena, 1990).

Cells expressing cell surface glycosyltransferase activity have previously been identified. As a source of sialyltransferase activity Cerven has reported such activity on the surface of intact Ehrlich ascites cells that were passed in Swiss albino mice. Bernacki has also measured endogenous sialyltransferase activity on intact leukemic L-1210 cells. For a review of cell surface glycosyltransferase activity, see Pierce et al., International Review of Cytology, 65: 1-44 (1980).

In other cases it has been recognized that some glycosyltransferases, particularly those which are membrane bound require the presence of an additional protein to exhibit transferase activity (Kelleher,D.J. et al, Cell, 69: 55-65, 1992)).

Further, methods have been developed to alter the glycosyltransferases expressed by cells. Larsen et al., Proc. Natl. Acad. Sci. U.S.A., 86: 8227-8231 (1989), report a genetic approach to isolate cloned cDNA sequences that determine expression of cell surface oligosaccharide structures and their cognate glycosyltransferases. A cDNA library generated from mRNA isolated from a murine cell line known to express UDP-galactose:β-D-galactosyl-1,4-N-acetyl-D-glucosaminide α-1,3-galactosyltransferase was transfected into COS-1 cells. The transfected cells were then cultured and assayed for α 1-3 galactosyltransferase activity.

Paulson et al., U.S. Patent No. 5,032,519, discloses a method of producing secretable glycosyltransferases.

According to this method, eukaryotic cells express a genetically altered soluble form of a glycosyltransferase in addition to the endogenous Golgi-bound form of the enzyme. However, the Paulson et al method is limited only to eukaryotic cell systems.

Francisco et al, Proc. Natl. Acad. Sci. U.S.A., 89: 2713-2717 (1992), disclose a method of anchoring β-lactamase to the external surface of *Escherichia coli*. A tripartite fusion consisting of (i) a signal sequence of an outer membrane protein, (ii) a membrane-spanning section of an outer membrane protein, and (iii) a complete mature β-lactamase sequence is produced resulting in an active surface bound β-lactamase molecule. However, the Francisco method is limited only to procaryotic cell systems and as recognized by the authors, requires the complete tripartite fusion for proper functioning. Such bacterial tripartite fusions may not be suitable for industrial purposes because of the extreme burden on a cell to produce the long fusion protein thereby reducing cellular efficiency and growth. Production of the fusion protein construct is believed to be counter productive.

Despite the advancements in modulation of bound and unbound glycosyltransferases, the applications of such modified organisms has been very limited. In fact, these transformed cells have only been used to transgenically produce glycosylated proteins where only the non-glycosylated proteins have previously been available.

Since extracellular glycosyltransferases appear on the cell surface, it is now possible to utilize the activity of these glycosyltransferases in a synthetic method.

Disclosure of the Invention

Accordingly, one object of this invention is to provide a novel method of synthesizing saccharide compositions, including oligosaccharides, using cell surface bound glycosyltransferases.

Another object of the present invention is to provide a bioreactor suitable for synthesizing said saccharide

compositions in accordance with the invention comprising at least one cell culture (if more than one, then each expressing a different glycosyltransferase) and means for isolating the saccharide composition.

Another object of the present invention is to provide a bioreactor suitable for synthesizing said saccharide compositions in accordance with the invention comprising at least one cell culture, expressing a glycosyltransferase along with the conjugate donor saccharide.

The inventor has now discovered that the above objects of the invention, and other objects which will become apparent from the description of the invention given hereinbelow, are satisfied by a method for the glycosyltransferase catalyzed preparation of a saccharide composition by serially bonding preselected saccharide units to an acceptor moiety in which (i) an acceptor moiety is contacted with at least one donor saccharide in the presence of at least one cell surface-bound glycosyltransferase specific for catalyzing the coupling of the acceptor moiety with the donor saccharide. The acceptor moiety is a carbohydrate, a glycoprotein, or a glycolipid. When the acceptor moiety is a protein or lipid, the resulting product is an O-linked glycoprotein or an O-linked glycolipid. The saccharide composition product is then isolated, and optionally further purified.

Best Mode for Carrying Out the Invention

As employed in this text, the term "saccharide composition" includes any chemical moiety having a saccharide unit within its structure. Sugars, carbohydrates, saccharides, monosaccharides, oligosaccharides, polysaccharides, glycoproteins, and glycolipids are examples of saccharide compositions. Mixtures and solutions comprising such entities are also saccharide compositions.

In accordance with the present invention, an acceptor moiety is provided which is capable of being covalently bound to a preselected saccharide unit. Representative acceptor moieties include proteins, glycoproteins, lipids, glycolipids,

and carbohydrates such as monosaccharides, disaccharides, oligosaccharides and/or polysaccharides. It will be appreciated that acceptor moieties are preferred to the extent that they are present as a structural component of a saccharide composition of interest. It will be appreciated that when proteins and lipids are the acceptor, an O-linked glycoprotein or O-linked glycolipid will result. In order to form N-linked glycoproteins, the core saccharide unit must first be attached. For example, in preparing a saccharide composition such as N-acetylneuraminyl α 2-3 galactosyl β 1-4 N-acetylglucosamine, the acceptor moieties are N-acetylglucosamine and galactosyl β 1-4 N-acetylglucosamine. It will likewise be appreciated that where an acceptor moiety is terminated by a saccharide unit, subsequent saccharide units will typically be covalently bound to the nonreducing terminus of the terminal saccharide.

The donor saccharide is provided in the form of a nucleoside mono- or diphosphate sugar. In mammalian systems, 8 monosaccharides are activated in this form to provide the building blocks for most oligosaccharides: UDP-Glc, UDP-GlcUA, UDP-GlcNAc, UDP-Gal, UDP-GalNAc, GDP-Man, GDP-Fuc and CMP-NeuAc.

In its simplest form, the method of the present invention provides for bringing together an acceptor and at least one donor saccharide in the presence of at least one cell culture. When more than one cell culture is used, each may preferably bear a different cell surface bound glycosyltransferase capable of catalyzing the coupling of the acceptor and one donor saccharide as well as the acceptor-donor saccharide complex with the second donor saccharide. A single donor saccharide may be used where the trisaccharide is the result of binding the acceptor with two units of the same donor saccharide. The cell culture is allowed to grow and continually produce cells bearing membrane-bound glycosyltransferase. In the presence of an acceptor and two donor saccharides, a bioreactor for producing a trisaccharide is provided.

The cells used according to the present process are cultures of a cell which expresses, on the cell surface, the glycosyltransferase of interest which is capable of catalyzing the reaction between the acceptor and donor moieties. While cell surface glycosyltransferases occur naturally, it is also possible to transfect cells with genes to express a glycosyltranferase of interest.

Transfected cells may be obtained by methods known to those of ordinary skill in the art. For example, to obtain transfected cells, DNA, including cDNA, that contain the natural or modified sequences, that encode the catalytic and transmembrane regions of the glycosyltransferase, are transferred into a cell which naturally lacks expression of the glycosyltransferase of interest. Cells that already have cell surface expression of the glycosyltransferase of interest may also be transfected with the desired cDNA in order to obtain cells that have an even higher specific transferase activity on their surface.

In many cases DNA (including cDNA) encoding glycosyltransferase genes have already been isolated and sequenced. A gene that encodes lactose synthetase has been reported for the bovine enzyme (Narimatsu et al, Proc. Natl. Acad. Sci. USA, 83: 4720-24 (1986) and Shaper et al, Proc. Natl. Acad. Sci. USA, 83: 1573-77 (1986)) and a human enzyme (Appert et al, Biochem. Biophys. Res. Comm., 139: 163-68 (1986)). Other glycosyltransferase genes which have been reported are two sialyltransferase (Weinstein et al, J. Biol. Chem., 262: 17735-43 (1987); Wen et al.,J. Biol. Chem., 267: 21011-19 (1992)), rat liver glucuronyltransferase (Jackson and Burcheli, Nuc. Acids Res., 14: 779-95 (1986); Mackenzie, J. Biol. Chem., 261: 14112-17 (1986)), a mouse glucuronyltransferase (Kimura and Owens, Eur. J. Biochem., 168: 515-21 (1987)), a human glucuronyltransferase (Jackson, Biochem. J., 242: 581-88 (1987)), a human N-acetylgalactosaminyltransferase (Nagata et al., J. Biol. Chem., 267: 12082-89 (1992)), a mouse galactosyltransferase (Larsen et al., Proc. Natl. Acad. Sci. U.S.A., 86: 8227-31

(1989)), a rabbit N-acetylglucosaminyltransferase I (Sarkar et al., Proc. Natl. Acad. Sci. U.S.A., 88: 234-38 (1991)), a rat N-acetylglucosaminyltransferase III (Nishikawa et al., J.Biol. Chem., 267: 18199-204 (1992)), a number of human fucosyltransferases (Larsen et al., Proc. Natl. Acad. Sci. U.S.A., 87: 6674-78 (1990); Kukowska-Latallo et al., Genes and Development 4: 1288-303 (1990); Weston et al., J.Biol. Chem., 267: 4152-60 (1992)), a human N-acetylglucosaminyltransferase (Berhuizen and Fukuda, Proc. Natl. Acad. Sci. U.S.A., 89: 9326-30 (1992)), and a bovine N-acetylgalactosaminyltransferase (Homa et al., J. Biol. Chem., 268: 12609-16 (1993)).

For glycosyltransferases for which a gene (DNA or cDNA) is not readily available, the following approach may be used to obtain the DNA(s) encoding the glycosyltransferase(s) of interest. Generally, in the synthesis of a saccharide composition in accordance with the invention, a preselected saccharide unit is first enzymatically attached to an initial acceptor moiety, i.e., a protein, a glycoprotein, a lipid, a glycolipid, or a carbohydrate starting material. This is followed by enzymatically attaching preselected saccharide units to the product obtained in a sequential fashion thereby forming the saccharide composition.

With the attachment of each preselected saccharide unit, one obtains an intermediate product. As described in greater detail in the inventor's pending application serial no. 07/683,810, in the invention described therein the starting material of the synthesis (i.e., the protein, glycoprotein, lipid, glycolipid or carbohydrate) and each intermediate product formed in the synthesis can be advantageously used to obtain, for each corresponding step of the synthesis, a glycosyltransferase specific to catalyze the attachment of the next intermediate product in the synthesis of the target saccharide composition.

Thus, the glycosyltransferase needed for any given step is isolated with the intermediate product (the acceptor moiety) and used to attach to the acceptor moiety the next

saccharide unit necessary for construction of the target carbohydrate molecule. This process may be repeated, with each iteration (time) yielding the particular glycosyltransferase required to attach the next saccharide unit onto the growing molecule being isolated, until the target carbohydrate molecule is obtained. In this manner, glycosyltransferases specific for any particular acceptor moiety and capable of transferring a preselected saccharide unit to the acceptor moiety may be isolated and accordingly all of the enzymes for a given synthesis may be obtained.

Alternatively, the peptide sequence may be obtained from a glycosyltransferase that has been purified by conventional methods known to those of ordinary skill in the art. Synthetic degenerate oligonucleotides derived from the peptide sequence can be used to screen lambda, cosmid, or YAC libraries and thus isolate cDNA or genomic clones for the various glycosyltransferases. The polymerase chain reaction (PCR) method can also be used to clone glycosyltransferases using the synthetic oligonucleotide probes described above. Finally, expression cloning which has been used successfully to isolate a number of glycosyltransferases (Larsen st al., Proc. Natl. Acad. Sci. U.S.A., 87: 6674-78 (1990); Nagata et al., J. Biol. Chem., 267: 12082-89 (1992)) can also be used to obtain cDNAs encoding other glycoslytransferases.

Each requisite enzyme needed to synthesize an oligosaccharide of interest may be identified and obtained by contacting the acceptor moiety with a mixture suspected to contain a plurality of glycosyltransferases, including the glycosyltransferase of interest, under conditions effective to bind the acceptor moiety and the glycosyltransferase specific for the acceptor moiety. The mixture suspected to contain the glycosyltransferase of interest may be identified as follows. For the most common glycosidic linkages, the glycosyltransferase activities have been described in publications. This is largely true for compounds like milk oligosaccharides, or the carbohydrate moieties of typical (i.e., prevalent) glycoproteins and glycolipids. For less

well described linkages, one may first look to the tissue, organ, on foodstuff organism, in which the linkage is found. Generally, if the linkage is found in a particular source, the enzyme that made the linkage is also present in the source.

If one is presented only with a saccharide structure, and not a source, one can then test examples of organisms that are likely to contain such a saccharide structure (relying on publications of relevant structures) using the most sensitive screening assay available. For example, if the target compound contained iduronic acid, N-acetylgalactosamine and N-acetylglucosamine, one would test vertebrate connective tissue. If the target compound contain abequose, one would test bacteria and plants for the presence of the appropriate glycosyltransferase.

Various assays for detecting glycosyltransferases which can be used in accordance with the invention have been published. The following are illustrative. Furukawa et al, Biochem. J., 227:573-582 (1985) describe a borate-impregnated paper electrophoresis assay and a fluorescence assay (figure 6) developed by the inventor. Roth et al, Exp'l Cell Research 143:217-225 (1983) describe application of the borate assay to glucuronyl transferases, previously assayed colorimetrically. Benau et al, J. Histochem. Cytochem. , 38:(1):23-30 (1990) describe a histochemical assay based on the reduction, by NADH, of diazonium salts.

Once a source for the glycosyltransferase of interest has been found, the source is homogenized. The enzyme is purified from the homogenate by affinity chromatography using the acceptor moiety as the affinity ligand. That is, the homogenate is passed over a solid matrix having immobilized thereon the acceptor moiety under conditions which cause the glycosyltransferase to bind to the acceptor moiety.

Monitoring for acceptor-bound enzyme can be carried out as follows. The cell homogenate is passed over the immobilized acceptor moiety. This may be achieved, for example, by passing the cell homogenate over a column charged with immobilized acceptor moiety. The column is then washed

and the amount of protein which passes through the column charged with immobilized acceptor moiety is monitored. When no more protein is detected, an aqueous salt solution or solution of a suitable sugar donor eluant is passed through the column to elute the enzyme. The eluant obtained is then assayed for the presence of glycosyltransferase(s). The assays which can be used are noted above, i.e., the methods described by Furukawa et al., Roth et al and Benau et al.

If no binding of the enzyme to the acceptor moiety occurs (i.e., the assay of the eluate fails to reveal the presence of glycosyltransferase(s) therein), then it can be concluded that the mixture did not contain an enzyme specific for the particular acceptor. Other mixtures of, for example, animal and/or plant cell homogenates are then contacted with the acceptor moiety until enzyme binding is observed.

When the acceptor moiety is bound by an enzyme, the species are separated. For example, the solid support matrix having the glycosyltransferase bound thereto is washed. This is followed by an elution step in which the glycosyltransferase is desorbed from the solid support matrix and collected. As known, the absorbed glycosyltransferase may be eluted, for example, by passing an aqueous salt (e.g. NaCl) solution over the solid support matrix.

In a preferred embodiment, the acceptor and the candidate enzyme are again contacted, this time in the presence of a donor moiety which comprises the saccharide unit desired to be transferred to the acceptor moiety. If such contacting results in the transfer of the saccharide unit to the acceptor, the enzyme is a glycosyltransferase useful in the practice of this invention.

It will be appreciated that once the glycosyltransferase is identified and isolated, it can be sequenced, DNA sequence encoding the enzyme obtained and/or replicated by techniques well-known to those skilled in the art. For example, obtaining a DNA sequence encoding the enzyme may be accomplished by recombinant techniques involving the isolation of genetic material coding for the glycosyltransferase and the

preparation of an immortal cell line capable of amplifying the amount of DNA encoding the glycosyltransferase. This may be accomplished by determining the amino acid sequence of the glycosyltransferase (using known techniques) to a degree sufficient to enable isolation of a DNA sequence encoding the enzyme using a DNA or an RNA probe, preferably using degenerate probes.

In accordance with the present invention the DNA (including cDNA) encoding for glycosyltransferase genes can be introduced into a host cell in either native or engineered form in both procaryotic or eukaryotic cells using known techniques as follows.

In the case of procaryotes, the signal and transmembrane sequences of the glycosyltransferase are replaced by a bacterial signal sequence, capable of effecting localization of the fusion protein to the outer membrane. Suitable signal sequences include, but are not limited to those from the major *E.coli* lipoprotein Lpp and lam B. In addition, membrane spanning regions from Omp A, Omp C, Omp F or Pho E can be used in a tripartite fusion protein to direct proper insertion of the fusion protein into the outer membrane. Any procaryotic cells can be used in accordance with the present invention including but not limited to *E.coli*, *Bacillus sp.*, and *Pseudomonas sp.* as representative examples.

In another embodiment, the native transmembrane domain of the glycosyltranferase is replaced by the transmembrane domain of a bacterial outer membrane protein. In this embodiment, the glycosyltransferase signal sequence and the bacterial transmembrane region act in concert to anchor the glycosyltransferase to the bacterial outer cell membrane. Any outer membrane bound protein is suitable for this use including but not limited to Omp A, Omp C, and Omp F, Lpp, and Lam B so long as the transmembrane structure is known to such extent that one can determine the position, in the linear sequence, an extracellular loop occurs in the protein. The catalytic portion of the glycosyltransferase should be fused to an extracellular loop in the bacterial transmembrane region

in order to insure proper orientation of the fusion protein on the outer membrane surface and not in the cytoplasm or periplasm of the cell. Insertion into such a loop region without description of the proper transmembrane folding has been previously reported (Charbit et al., J.Bacteriology, 173: 262-275 (1991); Francisco, et al., Proc. Natl. Acad. Sci. U.S.A., 89: 2713-2717 (1992)).

The present invention is also applicable for use with eukaryotic cells resulting in cell surface expression of glycosyltransferases in known culturable eucaryotic cells including but not limited to yeast cells, insect cells, chinese hamster ovary cells (CHO cells), mouse L cells, mouse A9 cells, baby hamster kidney cells, C127 cells, and PC8 cells.

Paulson et al. (U.S. Patent No. 5,032,519, incorporated herein by reference) describe a method of engineering glycosyltranferases to result in secretion of a soluble form of the enzyme. The patentees describe the removal of the hydrophobic transmembrane anchor region of the glycosyltransferase which results in secretion of the protein in a soluble form. In the present invention the native transmembrane region of the glycosyltransferase is modified to allow the recombinant protein to be localized to the extracellular surface of the plasma membrane of the cell.

In a preferred embodiment of the present invention, the transmembrane domain of the glycosyltransferase is replaced by the transmembrane domain of a plasma membrane protein. The transmembrane domain of any resident plasma membrane protein will be appropriate for this purpose. The transmembrane portions of the M6P/IGF-II receptor, LDL receptor or the transferrin receptor are representative examples. Further, a short cytoplasmic peptide in addition to the transmembrane portion would give improved anchoring into a plasma membrane. The cytoplasmic tails of any of the three previously mentioned receptor proteins will suffice if their internalization signals have been inactivated by site-directed mutagenesis (Johnson et al., Proc. Natl. Acad. Sci. U.S.A., 87: 10010-

10014 (1990); Canfield et al., J.Biol.Chem., 266: 5682-5688 (1991)). Inactivation of the internalization signal can be achieved by modification of the four amino acid sequence tyr-X-X-Y, where X is any amino acid and Y is either leu, isoleu or val. The internalization signal is inactivated by conversion of the tyrosine to an alanine and Y to an alanine.

In another preferred embodiment the Golgi retention signal of the glycosyltransferase is disrupted by site-directed mutagenesis. This approach mutates the few amino acids responsible for localizing the glycosyltransferase to the Golgi compartment. The resultant glycosyltransferase is transported to the plasma membrane where it becomes anchored via its modified transmembrane sequences. Substitution of isoleucine residues for the native amino acids in the transmembrane region of the β-1, 4-galactosyltransferase has been shown to preferentially localize the enzyme to the plasma membrane instead of the Golgi apparatus (Masibay et al., J.Biol.Chem., 268: 9908-9916 (1993)). While not wishing to be bound to any particular theory, it is believed that substitution of the isoleucine residues, increases the hydrophobicity of the transmembrane sequence, resulting in the preferential localization of the enzyme in the plasma membrane.

Any of the well known procedures, to those of ordinary skill in the art, for introducing foreign DNA sequences into a host cell may be used with the present invention. Suitable vectors for transporting the DNA into the cell include but are not limited to plasmid vectors, viral vectors and any of the other well known methods for introducing cloned genomic DNA, cDNA, synthetic DNA, semi-synthetic DNA and any other foreign genetic materials into a host cell.

Suitable procaryotic vectors include but are not limited to pBR322, pMB9, pUC, lambda bacteriophage, m13 bacteriophage, and Blue script®.

Suitable eukaryotic vectors include but are not limited to pMSG, pAV009/A+, PMTO10/A+, pMAM neo-5, bacculovirus, pDSVE, YIP5, YRP17, YEP.

It will be clear to one of ordinary skill in the art which vector or promoter system should be used depending on which cell type is used for a host cell.

Preferably, the cell culture only expresses the cell surface glycosyltransferase of interest.

Suitable cells may be identified by incubating the cells believed to express the cell surface glycosyltransferase activity of interest with the appropriate acceptor and radiolabeled donor sugar moiety. At the end of the incubation period, the glycosylated product is separated from the unused radioactive substrate. The synthesis of the appropriate acceptor-donor condensate is then evidence of the presence of the glycosyltransferase activity of interest on the cell surface.

In this fashion, an entire library of cell cultures may be identified which express, on their cell surface, a specific glycosyltransferase. For example, a cell culture which expresses N-acetylglucosaminyl transferase on the cell surface may be identified and stored. Likewise a cell culture which expresses a galactosyltransferase may also be identified and stored.

Once a library of suitable glycosyltransferases has been identified, it is then possible to select the specific glycosyltransferases needed to synthesize an oligosaccharide of interest.

For example, in order to synthesize lacto-N-neotetraose, an oligosaccharide found in human milk of the structure β-D-Gal 1-4 β-D-GlcNAc 1-3 β-D-Gal 1-4 D-Glc, it is necessary to provide two cell cultures which provide the requisite two glycosyltransferases necessary to form the β-Gal 1-4 GlcNAc bond, and the β-GlcNAc 1-3 Gal bond. In the presence of these two cell cultures and UPD-GlcNAc, and UDP-Gal , β-D-Gal 1-4 D-Glc(i.e. lactose) is converted into lacto-N-neotetraose.

By assembling the cell cultures, acceptor and source of donor sugars, it is now possible to design a bioreactor to synthesize any oligosaccharide composition containing naturally occurring glycosidic linkages. The bioreactor would

mimic the results of a conventional bioreactor, in that cultured cells would produce a biological product of interest, however, in the present process, the product of interest can be determined at will. The bioreactor may contain any number of different cell cultures depending on the number of glycosyltransferases needed to prepare the oligosaccharide of interest. Particularly the bioreactor may contain from one, two, three or four different cell cultures, each expressing a different glycosyltransferase on the cell surface. The separate cell cultures will provide the same function as a single cell, however since the cell cultures and donor sugars may be selected, the resulting product may be predetermined.

Suitable cells which express glycosyltransferase activity may be transfected procaryotic or eukaryotic cells. Preferably, transfected yeast cells which express the glycosyltransferases of interest are used.

The present invention may also be practiced with a multiplicity of cells expressing different glycosyltransferases in the same manner as described above, such that a cell surface glycosyltransferase and conjugate donor saccharides are contacted with a suitable acceptor to form an oligosaccharide.

In this fashion, any oligosaccharide of interest may be formed simply by bringing together the appropriate cells expressing the requisite cell surface glycosyltransferases and the necessary acceptor and donor saccharides. It is now possible to generate a "designer" bioreactor through selection of cell cultures and donor saccharide units.

In a preferred embodiment, a bioreactor containing a cell culture that expresses a given conjugate sugar nucleotide donor, due to transfection of cDNAs that encode the enzymes necessary for synthesis of this saccharide donor, is allowed to interact with another bioreactor containing a cell culture which has surface expression of the appropriate glycosyltransferase for the conjugate sugar nucleotide. Suitable cells are identified by including the appropriate radiolabeled acceptor moiety in the culture medium. At the

end of the incubation period, the glycosylated product is separated from the unused radioactive acceptor. Formation of the radiolabeled product is evidence that a cell culture expressing the conjugate saccharide donor and a cultured cell expressing the glycosyltransferase on its surface are both present. It is also possible that the cell culture which has surface expression of the appropriate glycosyltransferase and the cell culture which expresses a given conjugate sugar nucleotide donor interact in a single bioreactor.

In another preferred embodiment, the same cell culture has both surface expression of a glycosyltransferase and expresses the conjugate donor saccharide. Suitable cells are identified by incubating the cell believed to express the cell surface glycosyltransferase and conjugate moiety. At the end of the incubation period, the glycosylated product is separated from the unused radioactive acceptor. The reaction of the radioactive acceptor is evidence of the presence of a surface glycosyltransferase and conjugate donor saccharide in the same cell culture. The glycosyltransferase activity is then identified by characterization of the acceptor-donor saccharide composition.

Through identification of cell cultures which express both a glycosyltransferase and conjugate donor saccharide, the efficiency of any bioreactor containing this cell culture is greatly enhanced due to obviating the need to separately supply the donor saccharide.

The bioreactor according to the present invention may be any conventionally used cell culture reactor. A suitable reactor will provide a means for maintaining a stable suitable temperature, a supply of essential cell nutrients, and means for mixing the contents of the reactor.

According to the present process, cells may be cultured continuously, or in batches according to standard techniques.

Cells may be cultured in a modified batch process through continuous phasing of the nutrient environment (U.S. 3,419,703)

The saccharide compositions which can be prepared in

accordance with this invention are believed to find wide utility in diagnostics, therapeutics, and pharmacological applications. Once the sugar sequence of a desired target saccharide composition has been determined by conventional methods, a retrosynthetic analysis is generally performed to determine an appropriate synthetic scheme for the saccharide composition. Such a synthetic scheme preferably identifies the particular donor moieties, acceptor moieties, and glycosyltransferase necessary to yield the desired saccharide composition.

* * *

These and other features of the invention will become apparent in the course of the following descriptions of exemplary embodiments which are given for illustration of the invention and are not intended to be limiting thereof.

* * *

Example 1

Synthesis of lacto-N-neotetraose

To a cell culture apparatus containing a suitable reaction culture medium is added two cell cultures, each of which express a different glycosyltransferase. One cell culture expresses a glycosyltransferase necessary to produce the Gal 1-4 GlcNAc bond. A second cell culture provides a glycosyltransferase necessary to form the GlcNAc 1-3 Gal bond. The reaction medium consists of water and suitable nutrients to support the growth of the cell culture. Into this cell culture apparatus is also provided, one eq. of UDP-GlcNAc, and one eq. of UDP-Gal. A molar equivalent of lactose is then added to the reaction medium. The reactor is brought to a temperature of 35°C and reacted for 28 hours. The cell cultures are then separated by filtration and the supernatant liquid is purified by chromatography to yield lacto-N-neotetraose.

* * * * *

Obviously, numerous modifications and variations of the present invention are possible in light of the above teachings. It is therefore to be understood that within the

scope of the appended claims, the invention may be practiced otherwise than as specifically described herein.

Claims

1. A method for the glycosyltransferase-catalyzed preparation of a saccharide composition by serially bonding preselected saccharide units to an acceptor moiety comprising:

(i) contacting an acceptor moiety with at least one donor saccharide in the presence of at least one cell surface-bound glycosyltransferase specific for catalyzing the coupling of the acceptor moiety with said donor saccharide, wherein said acceptor moiety is a carbohydrate, a protein, a glycoprotein, a lipid, or a glycolipid, and

(ii) isolating said saccharide composition.

2. The method of Claim 1, wherein at least two different cell surface bound glycosyltransferases and two donor saccharides are used.

3. The method of Claim 1, wherein said cell is a procaryote.

4. The method of Claim 3, wherein said acceptor moiety is a carbohydrate.

5. The method of Claim 4, wherein said carbohydrate is a monosaccharide.

6. The method of Claim 4, wherein said carbohydrate is a disaccharide.

7. The method of Claim 4, wherein said carbohydrate is an oligosaccharide.

8. The method of Claim 4, wherein said carbohydrate is a polysaccharide.

9. The method of Claim 3, wherein said acceptor moiety is a protein.

10. The method of Claim 3, wherein said acceptor moiety is a glycoprotein.

11. The method of Claim 3, wherein said acceptor moiety is a lipid.

12. The method of Claim 3, wherein said acceptor moiety is glycolipid.

13. The method of Claim 1, wherein said cell is a eukaryote.

14. The method of Claim 13, wherein said acceptor moiety is a carbohydrate.

15. The method of Claim 14, wherein said carbohydrate is a monosaccharide.

16. The method of Claim 14, wherein said carbohydrate is a disaccharide.

17. The method of Claim 14, wherein said carbohydrate is an oligosaccharide.

18. The method of Claim 14, wherein said carbohydrate is a polysaccharide.

19. The method of Claim 13, wherein said acceptor moiety is a protein.

20. The method of Claim 13, wherein said acceptor moiety is a glycoprotein.

21. The method of Claim 13, wherein said acceptor moiety is a lipid.

22. The method of Claim 13, wherein said acceptor moiety

is a glycolipid.

23. The method of Claim 1, wherein said cell is a transfected cell which expresses β1-4 glycosyltransferase.

24. The method of Claim 1, wherein said cell is a transfected cell which expresses β1-3 glycosyltransferase.

25. The method of Claim 1, wherein said cell is a yeast cell.

26. The method of Claim 3, wherein said cell is transfected with a DNA vector comprising cDNA encoding for a glycosyltransferase.

27. The method of Claim 26, wherein the native signal sequence of said cDNA has been replaced with a bacterial signal sequence.

28. The method of Claim 27, wherein said signal sequence comprises a defective signal peptidase cleavage site.

29. The method of Claim 27, wherein said bacterial signal sequence is a protein selected from the group consisting of OmpA, Lpp, and LamB.

30. The method of Claim 26, wherein said cDNA additionally comprises a portion of DNA encoding for a hydrophobic protein region, wherein said DNA segment is inserted after the native signal sequence in said cDNA.

31. The method of Claim 30, wherein said DNA or sequence encodes for the hydrophobic region of a transmembrane protein.

32. The method of Claim 31, wherein said hydrophobic region of a transmembrane protein is selected from the group consisting of OmpA, Lpp and LamB.

33. The method of Claim 13, wherein said cell is transfected with a cDNA containing DNA vector comprising a native signal sequence, and a region of DNA encoding for a glycosyltransferase, wherein the DNA encoding for the hydrophobic membrane region has been deleted.

34. The method of Claim 33, wherein said signal sequence comprises a defective signal peptidase cleavage site.

35. The method of Claim 33, wherein said cDNA further comprises a section of DNA encoding for a segment of a transmembrane protein.

36. The method of Claim 35, wherein said segment of DNA encodes for a transmembrane protein selected from the group consisting of OmpA, Lpp, and LamB.

37. The method of Claim 1, wherein the carboxyterminus of said glycosyltransferase is C-terminus bound to said cell surface.

38. The method of Claim 28, when said signal peptidase cleavage site has been destroyed by site-directed mutagenesis of the DNA encoding said signal sequence.

39. The method of Claim 34, wherein the carboxyterminus of said glycosyltransferase is C-terminus bound to said cell surface.

40. A bioreactor suitable for the glycosyltransferase catalyzed preparation of a saccharide composition by serially bonding preselected saccharide units to an acceptor moiety, comprising:

a reaction means comprising at least one culture of cells which expresses a cell surface membrane bound glycosyltransferase, and a source of a donor saccharide moiety specific for said membrane bound glycosyltransferase, and

means for isolating said saccharide composition.

41. A bioreactor suitable for the glycosyltransferase catalyzed preparation of a saccharide composition by serially bonding preselected saccharide units to an acceptor moiety, comprising:

a reaction means comprising at least two cell cultures of cells, each of which express a different cell surface membrane bound glycosyltransferase and conjugate donor saccharide, and

means for isolating said saccharide composition.

42. The bioreactor of Claim 40, wherein said cell cultures are transfected cells, transfected to express a glycosyltransferase

43. The bioreactor of Claim 40, wherein one of said cell cultures is a culture of transfected cells which express β1-4 glucosyltransferase.

44. The bioreactor of Claim 40, wherein one of said cell cultures is a culture of transfected cells which express β1-3 galactoslytransferase.

45. The bioreactor of Claim 40, wherein said cells are yeast cells.

46. The bioreactor of Claim 40, wherein said cells are procaryotes.

47. The bioreactor of Claim 46, wherein said cells are transfected with a DNA vector comprising cDNA encoding for a glycosyltransferase.

48. The bioreactor of Claim 46, wherein the native signal sequence of said cDNA has been replaced with a bacterial signal sequence.

49. The bioreactor of Claim 48, wherein said signal sequence comprises a defective signal peptidase cleavage site.

50. The bioreactor of Claim 48, wherein said bacterial signal sequence is a protein selected from the group consisting of OmpA, Lpp, and LamB.

51. The bioreactor of Claim 46, wherein said cDNA additionally comprises a portion of DNA encoding for a hydrophobic protein region, wherein said DNA segment is inserted after the native signal sequence in said cDNA.

52. The bioreactor of Claim 51, wherein said DNA or sequence encodes for the hydrophobic region of a transmembrane protein.

53. The bioreactor of Claim 51, wherein said hydrophobic region of a transmembrane protein is selected from the group consisting of OmpA, Lpp and LamB.

54. The bioreactor of Claim 40, wherein said cells are eukaryotic cells.

55. The bioreactor of Claim 54, wherein said cells are transfected with a cDNA containing DNA vector comprising a native signal sequence, and a region of DNA encoding for a glycosyltransferase, wherein the DNA encoding for the hydrophobic membrane region has been deleted.

56. The bioreactor of Claim 55, wherein said signal sequence comprises a defective signal peptidase cleavage site.

57. The bioreactor of Claim 55, wherein said cDNA further comprises a section of DNA encoding for a segment of a transmembrane protein.

58. The bioreactor of Claim 57, wherein said segment of

DNA encodes for a transmembrane protein selected from the group consisting of OmpA, Lpp, and LamB.

59. The bioreactor of Claim 40, wherein the carboxyterminus of said glycosyltransferase is C-terminus bound to said cell surface.

60. The bioreactor of Claim 49, when said signal peptidase cleavage site has been destroyed by site-directed mutagenesis of the DNA encoding said signal sequence.

61. The bioreactor of Claim 56, wherein the carboxyterminus of said glycosyltransferase is C-terminus bound to said cell surface.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/07807

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/68.1, 69.8, 71.1, 72, 74, 85, 97, 100, 101, 193, 240.1, 252.3, 255, 320.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS search terms: glycosyl-, galactosyl-, fucosyl-, sialyl-transferase, cell-surface

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, Volume 67, Number 1, issued 1975, Patt et al, "Ectoglycosyltransferase Activity In Suspensions And Monolayers Of Cultured Fibroblasts", pages 483-490, see paragraph bridging pages 487-488, and page 488, paragraph 2.	1,2,13,20, 37,40,54, 59
X	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, Volume 59, Number 2, issued 1974, Yogeeswaran et al, "Mechanism Of Cell Contact-Dependent Glycolipid Synthesis: Futher Studies With Glycolipid-Glass Complex", pages 591-599, see page 594, Table 1, and page 595, paragraph 1.	1,13,21,22,37, 40,59
X	US, A, 4,590,160 (NISHIHASHI ET AL) 20 May 1986, see column 2, lines 25-62.	1-5,23,24, 40,46

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/07807

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US, A, 5,135,854 (MACKAY ET AL) 04 August 1992, see column 6, lines 31-64, and paragraph bridging columns 10-11.	1,2,13,19, 20,25,40, 42,45,54
X	US, A, 5,149,640 (OONISHI ET AL) 22 September 1992, see column 2, lines 37-60.	1,3-8,40,46
Y	THE JOURNAL OF BIOLOGICAL CHEMISTRY, Volume 267, Number 6, issued 25 February 1992, Teasdale et al, "The Signal for Golgi Retention of Bovine β1,4-Galactosyltranferase Is in the Transmembrane Domain", pages 4084-4096, see page 4088, column 2, paragraph 1, and page 4093, column 1, paragraph 3.	40,42,43, 54,59
Y,P	US, A, 5,324,663 (LOWE) 28 June 1994, see column 14, line 31 to column 15, line 9.	1-61
A	US, A, 5,032,519 (PAULSON ET AL) 16 July 1991, see abstract.	1-61
A	US, A, 5,047,335 (PAULSON ET AL) 10 September 1991, see abstract.	1-61
A	THE JOURNAL OF BIOLOGICAL CHEMISTRY, Volume 264, Number 30, issued 25 October 1989, Paulson et al, "Glycosyltransferases", pages 17615-17618, see page 17615, column 1, paragraph 1.	1-61

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(54) Title: A PROCESS FOR SYNTHESIZING OLIGOSACCHARIDES

(57) Abstract

The present invention relates to a process for synthesizing oligosaccharides. The process involves contacting an acceptor moiety with unpurified sugar–nucleotides and/or unpurified glycosyltransferase to synthesize oligosaccharides.

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A PROCESS FOR SYNTHESIZING OLIGOSACCHARIDES

Cross Reference to Related Applications

The present application is related to the U.S. patent applications entitled, "Nutritional Formulations Containing Lacto-N-neoTetraose," and "Nutritional Formulations Containing Oligosaccharides," all of which are filed concurrently herewith, and the texts of which are herein incorporated by reference.

Technical Field of the Invention

The present invention relates to a process for synthesizing oligosaccharide compositions. More specifically, the invention pertains to an economical method for synthesizing oligosaccharides using crude, unpurified sugar-nucleotides and/or glycosyltransferases.

Background of the Invention

The term "carbohydrate" embraces a wide variety of chemical compounds having the general formula $(CH_2O)_n$ and encompasses such compounds as monosaccharides, disaccharides, oligosaccharides, polysaccharides and their aminated, sulfated, acetylated and other derivated forms (U.S. Patent 5,288,637, this patent, as well as all other patents and publications disclosed herein are incorporated by reference). Oligosaccharides are chains composed of sugar units, which are also known as monosaccharides. Sugar units can be arranged in any order and linked by their sugar units in any number of different ways. *Id.* Therefore, the number of different stereoisomeric oligosaccharide chains possible is exceedingly large. *Id.*

Numerous classical chemical techniques for the synthesis of carbohydrates have been developed, but these techniques require selective protection and deprotection. Organic synthesis of oligosaccharides is further hampered by the lability of many glycosidic bonds, difficulties in achieving regioselective sugar

coupling, and generally low synthetic yields. Therefore, unlike peptide synthesis, traditional synthetic organic chemistry cannot provide for quantitative, reliable synthesis of even fairly simple oligosaccharides.

Recent advances in oligosaccharide synthesis have occurred with the characterization, cloning and isolation of glycosyltransferases. These enzymes can be used *in vitro* to prepare oligosaccharides, polysaccharides and other glycoconjugates. The advantage of biosynthesis with glycosyltransferases is that the glycosidic linkages formed by enzymes are highly stereo and regiospecific. Each enzyme catalyzes the linkage of specific sugar residues to other specific acceptor moieties, such as an oligosaccharide, lipid or protein. For example, U.S. Patent No. 5,288,637 discloses the stoichiometric synthesis of oligosaccharides using acceptor moieties, purified sugar-nucleotides and glycosyltransferases. The problem with this process, however, is that it is not commercially feasible due to the extremely high cost of the purified sugar-nucleotides. Therefore, a need exists for an economical process of synthesizing oligosaccharides using sugar-nucleotides and glycosyltransferases.

Summary of the Invention

The present invention relates to a process for synthesizing oligosaccharides *in vitro.* The process involves adding an acceptor moiety and a catalytic amount of a glycosyltransferase to a microorganism culture that produces a sugar-nucleotide or combinations of sugar-nucleotides and nucleotides. The mixture is maintained under conditions and for a period of time sufficient for oligosaccharide formation. Alternatively, the present invention also involves a process for synthesizing oligosaccharides by adding an acceptor moiety and intact host cells transformed with a polynucleotide that encodes a catalytic amount of a glycosyltransferase to sugar-nucleotides having a sugar unit to form a mixture and maintaining the mixture under conditions and for a period of time sufficient for oligosaccharide formation.

Detailed Description of the Invention

The present invention relates to a process for synthesizing oligosaccharides *in vitro*. The process involves adding an acceptor molecule and a glycosyltransferase to sugar-nucleotides under conditions and time sufficient to allow for oligosaccharide formation. The sugar-nucleotide and/or the glycosyltransferase used in the process of this invention are present in a crude, unpurified form. The use of the unpurified sugar-nucleotide and/or the unpurified glycosyltransferase makes the process of the present invention more economical than processes known and employed in the prior art that use purified sugar-nucleotides and purified or semipurified glycosyltransferases. (See U.S. Patent 5,288,637). The process of the present invention is amenable for use in a single or multiple batch operation, and the oligosaccharides produced, according to the process of the present invention, may be synthesized in a single or in multiple reaction vessels.

As known in the art, a monosaccharide is a sugar molecule that contains one sugar unit. As used herein, the term "sugar unit" means a monosaccharide. As also known in the art, a disaccharide is a sugar molecule that contains 2 sugar units, a trisaccharide is a sugar molecule that contains 3 sugar units, an oligosaccharide is a sugar molecule that contains between 2-10 sugar units, and a polysaccharide is a sugar molecule that contains greater than 10 sugar units. The sugar units in a di-, tri- and oligosaccharide are all connected by glycosidic linkages. Nonetheless, as used in the present invention, the term "oligosaccharide" means a sugar molecule that contains at least two sugar units.

The acceptor moiety employed in the present invention can be any molecule that is capable of being covalently bound to a sugar unit. Suitable acceptor moieties that can be used in this invention include, for example, proteins, glycoproteins, lipids, glycolipids, carbohydrates or any molecule having a sugar unit contained in its structure. The preferred acceptor moiety is a carbohydrate. The most preferred acceptor moiety is a mono-, di-, tri, or oligosaccharide. When the

acceptor moiety is terminated by a sugar unit, subsequent sugar units added to the molecule will typically be covalently bound to the nonreducing terminus of the end sugar of the molecule.

The sugar unit to be transferred to the acceptor moiety is provided by a sugar-nucleotide. In mammals, sugar-nucleotides are the building blocks for most oligosaccharides. Sugar-nucleotides are considered to be donor molecules since they provide sugar units to acceptor moieties during oligosaccharide synthesis. The sugar-nucleotides that may be used in the process of the present invention include, for example, saccharide terminated uridine mono- or di- phosphates, saccharide terminated guanosine mono- or di- phosphates and saccharide terminated cytidine mono- or di- phosphates. Examples of sugar-nucleotides that can be used in this invention include UDP-Glucose (UDP-Glc), UDP-N-acetylglucosamine (UDP-GlcNAc), UDP-Galactose (UDP-Gal), UDP-N-acetylgalactosamine (UDP-GalNAc), GDP-Mannose (GDP-Man), GDP-Fucose (GDP-Fuc) and CMP-N-acetylneuraminic acid (CMP-NeuAc).

Purified, semi-purified or unpurified sugar-nucleotides can be used in the present invention. As used herein, the term "purified and semi-purified sugar-nucleotides" refers to sugar nucleotides that have been processed in some manner, such as by ion-exchange chromatography or ultrafiltration, to increase their concentration and to separate the sugar-nucleotides from any other chemical compounds produced during the synthesis of said sugar-nucleotides. A dried, purified sugar nucleotide preparation is a preparation in which at least about 80% of the preparation's total weight is sugar nucleotides and no more than about 95% of the total weight of the preparation is sugar-nucleotides, counter-ions and water. For example, purified sugar nucleotides can be obtained by removing sugar nucleotides in a solution by precipitation, and then filtering the solution and then applying the solution to a selective ion exchange chromatography. The resulting solution is then lyophilized, or in the alternative, lyophilized and subjected to evaporation from ethanol or methanol to obtain purified sugar-nucleotides.

The term "semi-purified" as used herein refers to a sugar-nucleotide preparation where the actual residual content of the sugar-nucleotides in the preparation does not exceed 80% of the weight of the preparation. For example, semi-purified sugar nucleotides can be obtained by filtering a yeast solution containing sugar-nucleotides through a 10,000 M W filter and then drying the filtrate.

The term "unpurified" as used herein refers to sugar-nucleotides have not been processed in any manner, such as by ion-exchange chromatography or affinity chromatography, to increase their concentration and to separate the sugar-nucleotides from any chemical compounds produced during the biological or chemical synthesis of said sugar-nucleotides.

Sugar-nucleotides can be purified using any technique known in the art, such as by ion-exchange chromatography and ultrafiltration. See Smith, D., et al. Purified sugar-nucleotides are well known in the art and have frequently been employed in oligosaccharide synthesis. (Blake, D., et al., *Meth. Enzy.* 83 (1982) 127; Smith, D.F., *Meth. Enzy.* 83 (1982) 241).

Additionally, sugar-nucleotides produced by a microorganism culture can also be used in the present invention. The microorganism culture produces the sugar-nucleotides in a crude, unpurified form. Preferably, the sugar-nucleotides used in the process of this invention are in unpurified form and produced by a culture of microorganisms or by permeabilized or previously dried microorganisms.

The production of sugar-nucleotides using microorganisms, particularly yeast, is well known in the art *(See* Tochikura, T., et al., *J. Ferment. Technol.*, Vol. 46, No. 12, p. 957-969 (1968) and Tochikura, T. et al., *Agr. Biol. Chem.*, Vol. 35, No. 2, p. 163-176 (1971)). Generally, the microorganism culture can be prepared by incubating sugars at room temperature or in a refrigerator with microorganisms

and nucleotides or nucleotide precursors such as uridine mono- or di- phosphate, guanosine mono- or di- phosphate, or cytidine mono- or di- phosphate, orotate, in the presence of a phosphate source (such as phosphate or buffer), an energy source (such as glucose, fructose or maltose) and magnesium ions, for a sufficient amount of time to allow the microorganism to begin producing sugar-nucleotides. As used herein, the term "nucleotide precursor" refers to molecules that are used as intermediates in the synthesis of nucleotides via metabolic pathways, such as, nucleotides, purines or pyrimidines. Examples of sugar nucleotide precursors are uridine, orotate, citidine, adenosine, inosine, guanine, guanidine, guanosine, uridine, uridine mono-, di-, and tri-phosphates, citidine mono-, di- and tri-phosphates, guanosine mono-, di- and tri-phosphates. Precursors of purine synthesis, such as UMP and of UDP-sugar nucleotides, are: carbamoyl phosphate, aspartate, N-carbamoylaspartate, Dihydroorotate and orotidylate, other precursors are glycinamide ribonucleotide, formylglycinamide ribonucleotide, formylglycine ribonucleoitide, 5-aminonoimidazole ribonucleotide, 4-aminoimidazole-4crboxylate ribonucleotide 5-aminoimidazole-4-N-succinocarboxamide ribonucleotide, 5-aminoimidazole-4-carboxamideazole-4-carboxamide ribonucleotide, inosinate, adenylosuccinate, and xanthylate.

Any microorganism that is capable of producing sugar-nucleotides can be used in the present invention. For example, microorganisms of the genus *Saccharomyces, Zygosaccharomyces, Torulopsis, Candida, Crypococcus, Brettanomyces, Mucor, Hansenula* and *Debaryomyces* can be used. Examples of the strains of microorgansims that can be used to produce sugar-nucleotides are listed in Table 1.

Table 1

Saccharomyces
- *S. cerevisiae*
  - Baker's yeast (UDP-GlcNAc, UDP-Glc, GDP-Man)
  - Brewer's yeast (UDP-GlcNAc, UDP-Glc, GDP-Man)
- *S. fragilis* (UDP-Gal)
- *S. lactis* (UDP-Glc, UDP-Gal)
- *S. ludwigii* (UDP-GlcNAc)

Zygosaccharomyces
Z. rouxii (UDP-GlcNAc)

Torulopsis
T. candida (UDP-GlcNAc, UDP-Glc, UDP-Gal)
T. spaerica (UDP-Glc, UDP-Gal)
T. xlinus (GDP-Man)
T. versatilis (UDP-GlcNAc)

Candida
C. famata (UDP-GlcNac, UDP-Glc, UDP-Gal)
C. intermedia (UDP-Gal)
C. krusei (UDP-Glc)
C. parapsilosus (UDP-Glc)
C. utilis (GDP-Man)
C. mycoderma (UDP-Glc)
C. pseudostopicalis (UDP-Glc)
C. tropicalis (UDP-GlcNAc)

Crypococcus albidus (UDP-Glc)

Brettanomyces
B. anomalus (UDP-Glc, UDP-GlcNAc)
B. clausenii (UDP-Glc, UDP-Gal)

Mucor
M. javanicus (UDP-Glc)
M. racemosus (UDP-Glc)
M. circinelloides (UDP-Glc)

Hansenula
H. jadinii (GDP-Man)
H. saturnus (GDP-Man)
H. suaveolens (GDP-Man)
H. capsulata (UDP-Glc)

Debaryomyces
D. subglobosus (UDP-Glc, UDP-GlcNac)
D. globosus (UDP-GlcNac)
D. cantavellii (UDP-GlcNAc)
D. japonicus (UDP-GlcNAc)
D. hansenii (UDP-GlcNAc)

Preferably, the microorganisms used in this invention have been subjected to some type of processing such as drying, sonication, or solvent or detergent exposure. The preferred microorganism for use in this invention is *S. cerevisiae*, particularly, dried Baker's and dried Brewer's yeast, dried *Candida famata*, or *Zygosaccharomyces rouxii*.

In addition to the sugar-nucleotides, the microorganism culture or glycosyltransferase preparation may also contain one or more epimerases. An epimerse is an enzyme that changes the steroespecificity of hydroxyl groups at specific carbons in a saccharide. For example, an epimerase can be used to convert glucose to galactose and galactose to glucose. In the present invention, an epimerase may be used to convert UDP-Glc to UDP-Gal.

The crude, unpurified sugar-nucleotides described above are capable of providing component sugar units to an acceptor moiety when placed in contact with an acceptor moiety in the presence of at least one glycosyltransferase. As used herein, "glycosyltransferase" refers to an enzyme which facilitates the transfer of a sugar unit from one chemical entity (the donor molecule) to another (the acceptor moiety) and is named phenomenologically according to the sugar unit it transfers. For example, galactosyl-transferases transfer galactose and fucosyltransferases transfer fucose. Glycosyltransferases that can be used in this invention include, for example, fucosyltransferases, sialyltransferases, N-acetylglucose-aminyltransferases, galactosyltransferases, N-acetylgalactosaminyltransferases, glucosyl-transferases and mannosyltransferases.

Glycosyltransferases are known to possess three domains which correspond to three different areas of the gene encoding the enzyme. The area of the gene found at the 3' end is known to encode the catalytically functionally domain (Lowe, *Seminars in Cell Biology*, (1991) 2:289-307). The glycosyltransferases used in the process of this invention contain at least this catalytic domain, but may contain up to the whole protein sequence.

The glycosyltransferases used in this invention can be obtained from any source and can be in purified or crude, unpurified form. As used herein, a "purified" glycosyltransferase refers to a glycosyltransferase that has been processed in some manner, such as by affinity chromatography, to increase the specific activity of the enzyme. The term "specific activity" refers to the units per milligram of protein. Methods for purifying glycosyltransferases are well known in the art. An "unpurified" glycosyltransferase as used herein refers to a glycosyltransferase that has not been processed in any manner, such as by affinity chromatography, to increase the specific activity of the enzyme. Preferably, the glycosyltransferases used in the process of the present invention are in unpurified form.

Genes encoding glycosyltransferases and methods for producing recombinant molecules expressing glycosyltransferases are well known in the art. For example, genes encoding glycosyltransferases from *Neisseria gonorrhoeae* and recombinant molecules expressing these genes are described in WO 96/10086 and U.S. Patent No. 5,545,553.

Any gene encoding a glycosyltransferase can be inserted into a recombinant molecule. The polynucleotides constituting the gene may be obtained by standard procedures known in the art, such as from cloned DNA (such as a DNA "library"), chemical synthesis, cDNA cloning, or by the cloning of genomic DNA, or fragments thereof, from a desired cell as described in Sambrook, J., et al., Molecular Cloning A Laboratory Manual, 2d Edition, Cold Spring Harbor Laboratory Press (1989).

In the molecular cloning of the gene from genomic DNA, DNA fragments are generated, some of which will encode the desired gene. The DNA may then be cleaved at specific sites using various restriction enzymes. Alternatively, DNAse may be used in the presence of manganese to fragment the DNA, or the DNA can be physically sheared, as for example, by sonication. The linear DNA fragments

can then be separated according to size by standard techniques, such as by agarose and polyacrylamide gel electrophoresis and column chromatography.

Once the DNA fragments are generated, identification of the specific DNA fragment containing the desired glycosyltransferase gene may be accomplished in a number of ways that are well known in the art, such as through nucleic acid hybridization with one or more labeled probes as described in Sambrook, J., et al., Molecular Cloning A Laboratory Manual, 2d Edition, Cold Spring Harbor Laboratory Press (1989). The presence of the desired gene may then be detected using assays based on the physical, chemical, or immunological properties of the expressed product.

Once the gene encoding a glycosyltransferase has been isolated, it can be inserted into an appropriate cloning vector. A large number of vector-host systems known in the art may be used. Possible vectors include, but are not limited to, plasmids or modified viruses, provided that the vector system is compatible with the host cell used. The vectors that can be used include, for example, an *E. coli* cloning vector, bacteriophages such as lambda derivatives, plasmids such as pBR322 derivatives or pUC plasmid derivatives.

The insertion of the gene into the cloning vector can be accomplished by any process known in the art such as by ligating the DNA fragment into a cloning vector that has complementary cohesive termini. Sambrook, J., et al., Molecular Cloning A Laboratory Manual, 2d Edition, Cold Spring Harbor Laboratory Press (1989). However, if the complementary restriction sites used to fragment the DNA are not present in the cloning vector, then the ends of the DNA molecules may have to be enzymatically modified. Alternatively, any site desired may be produced by ligating nucleotide "linker" sequences onto the DNA termini. These ligated linkers may comprise specific chemically synthesized oligonucleotides encoding restriction endonuclease recognition sequences. The cloning vector can

be introduced into host cells via transformation, transfection, infection, electroporation, etc., so that many copies of the gene sequence are generated.

Transformation of host cells with a cloning vector that incorporates the glycosyltransferase gene enables the generation of multiple copies of the gene. Therefore, the gene may be obtained in large quantities by growing transformants, isolating the cloning vector from the transformants and, when needed, retrieving the inserted gene from the isolated cloning vector.

The cloning vector may contain genes encoding truncated forms of the enzyme (fragments) and derivatives of the gene that have the same functional activity as the full-length gene. A fragment or derivative is functionally active if it is capable of mediating transfer of a sugar unit to an acceptor moiety. For example, a cloning vector may contain a gene encoding the catalytically functional domain of a glycosyltransferase.

Once sufficient copies of the gene sequence have been generated, the gene encoding a glycosyltransferase, or a functionally active fragment or other derivative thereof, can be inserted into an appropriate recombinant molecule for use in the process of the present invention. The recombinant molecule is a polynucleotide expression vector that contains the necessary elements for the transcription and translation of the inserted protein-coding sequence. Preferably, the expression vector also includes an origin of replication. The necessary transcriptional and translation signals can also be supplied by the native glycosyltransferase gene and/or its flanking regions.

Once a recombinant molecule has been prepared, it is inserted into an acceptable host cell which will grow and divide to produce clones. A variety of host cell-vector systems may be utilized to express the gene. Suitable host cell-vector systems include, for example, bacterial expression systems, mammalian cell systems infected with a virus, such as a vaccinia virus or adenovirus, insect cell

systems infected with a virus such as a baculovirus, microorganisms such as yeast containing yeast vectors, and bacteria transformed with bacteriophage DNA, plasmid DNA or cosmid DNA. The preferred host cell-vector system for use in this invention is a bacterial cell expression system. The most preferred host cells for use in this invention are *E. coli* cells.

Any of the methods previously described for the insertion of DNA fragments into a vector may be used to construct expression vectors containing a chimeric gene consisting of appropriate transcriptional/translation control signals and the protein coding sequences. Expression of the polynucleotide encoding an glycosyltransferase or peptide fragment thereof may be regulated by a second nucleic acid sequence so that the glycosyltransferase or peptide fragment is expressed in a host transformed with the recombinant DNA molecule. For example, expression of a glycosyltransferase may be controlled by any promoter/enhancer element known in the art, but these regulatory elements must be functional in the host selected for expression. For expression in bacteria, bacterial promoters are required. Promoters which may be used to control glycosyltransferase gene expression include, for example, the SV40 early promoter region, the promoter contained in the 3' long terminal repeat of Rous sarcoma virus, the herpes thymidine kinase promoter, the regulatory sequences of the metallothionein gene; prokaryotic expression vectors such as the -lactamase promoter or the tac promoter.

Recombinant molecules containing the glycosyltransferase gene can be identified by PCR amplification of the desired plasmid DNA or specific mRNA, nucleic acid hybridization, presence or absence of marker gene functions and expression of the inserted sequences. Once a suitable host system and growth conditions are established, the recombinant molecules containing the glycosyltransferase gene can be introduced into the host cells via any procedure known in the art such as transformation, transfection, infection, electroporation, etc.

Once a source for the glycosyltransferases has been obtained, the source may be added directly to a reaction vessel for oligosaccharide synthesis. For example, host cells, such as *E. coli* cells, transformed with a polynucleotide that encode a crude, unpurified glycosyltransferase, can be added directly to a reaction vessel for use in the process of this invention. If the host cells express the glycosyltransferase into a culture medium, then the culture medium can be added to the reaction vessel. Additionally, the glycosyltransferase source can be homogenized and the homogenate added directly to a reaction vessel. If a purified glycosyltransferase is to be employed, the purified enzyme can be added directly to the reaction vessel. For example, if the glycosyltransferase source is homogenized, the glycosyltransferase can be purified from the homogenate by affinity chromatography using the acceptor moiety as the affinity ligand, using techniques known in the art.

In lieu of adding the unpurified or purified glycosyltransferase directly to the reaction vessel, the glycosyltransferase can instead be placed in a dialysis bag which can be inserted into a reaction vessel. The dialysis bag can be removed from the reaction vessel periodically and additional glycosyltransferases added when required. When the glycosyltransferase is contained in a dialysis bag, the acceptor moiety and sugar-nucleotides migrate into the bag and react with the glycosyltransferase to synthesize an oligosaccharide. When the synthesis of the oligosaccharide is completed, the oligosaccharide migrates out of the bag and into the surrounding medium.

The glycosyltransferases used in the present invention catalyze the transfer of sugar units from sugar-nucleotides to an acceptor moiety. The glycosyltransferases are preferably specific for a saccharide unit or at least some significant, active, or exposed portion thereof. Specificity is manifested for a glycosyltransferase by its tendency to bind with a particular sequenced portion of the acceptor moiety when placed in contact or close proximity therewith to effect the transfer of a particular sugar unit to the acceptor moiety.

A catalytic amount of a glycosyltransferase is employed in the process of the present invention. As used herein, a "catalytic amount" refers to the amount of glycosyltransferase that must be present to sufficiently catalyze the transfer of a sugar unit from a sugar-nucleotide to an acceptor moiety. The catalytic amount of glycosyltransferase employed in the process of the present invention can be determined by one of ordinary skill in the art through routine experimentation.

In the process of the present invention, an oligosaccharide can be synthesized by adding an acceptor moiety and a glycosyltransferase to a sugar-nucleotide to form a mixture and then maintaining the mixture under conditions and a period of time sufficient to allow for oligosaccharide formation. The conditions and time periods required for the formation of the oligosaccharide using the process of the present invention can be determined by one of ordinary skill in the art through routine experimentation. Generally, however, the mixture is incubated from about 4 to about 48 hours, at a temperature from about 4 to about 35 C and at a pH from about 4 to about 9.0.

In the process of the present invention, an oligosaccharide can be synthesized using a crude, unpurified sugar-nucleotide produced from microorganisms and a crude, unpurified glycosyltransferase produced from host cells transformed with a polynucleotide that expresses a glycosyltransferase. For example, an oligosaccharide can be synthesized by adding an acceptor moiety and *E. coli* cells transformed with a polynucleotide that encodes a catalytic amount of a glycosyltransferase to a culture of *S. cerevisiae* that produce sugar-nucleotides to produce a mixture. The mixture is then maintained under conditions and for a period of time sufficient to allow for oligosaccharide formation.

Alternatively, an oligosaccharide can be synthesized by adding an acceptor moiety and a catalytic amount of a purified glycosyltransferase to a culture of microorganisms producing sugar-nucleotides to form a mixture and incubating the mixture under conditions and for a period of time sufficient to allow for

oligosaccharide formation. Additionally, an oligosaccharide can be synthesized by adding an acceptor moiety and host cells transformed with a polynucleotide that encodes a catalytic amount of a glycosyltransferase to purified sugar-nucleotides to form a mixture and incubating the mixture under conditions and for a period of time sufficient to allow for oligosaccharide formation.

The oligosaccharide formed by the process of this invention can serve as an acceptor moiety for additional oligosaccharide synthesis. If further synthesis is required, the oligosaccharide and a catalytic amount of a glycosyltransferase are added to sugar-nucleotides to form another mixture. The mixture is maintained under conditions and for a period of time sufficient for oligosaccharide formation. This process is repeated until a sufficient number of sugar units have been transferred to form the desired oligosaccharide.

The synthesis of oligosaccharides according to the process of the present invention may take place in one or a number of reaction vessels. If a single reaction vessel is used, the ingredients required for oligosaccharide synthesis may be added sequentially, one at a time. For example, the ingredients required for the formation of a microorganism culture that produces the sugar-nucleotides may be added first followed by the acceptor moiety and then host cells transformed with a polynucleotide that encodes a glycosyltransferase. Depending on the oligosaccharide to be synthesized, additional sugar-nucleotides and glycosyltransferases may be added to the reaction vessel if necessary. Alternatively, all of the ingredients required for the synthesis of a particular oligosaccharide may be added to the reaction vessel at the same time.

A number of reaction vessels may also be used for the synthesis of oligosaccharides. For example, the ingredients required for the formation of a microorganism culture producing sugar-nucleotides, an acceptor moiety and host cells transformed with a polynucleotide that encodes a glycosyltransferase may be added sequentially or at the same time into a reaction vessel. The resulting oligosaccharide is then removed from the reaction vessel to a second reaction

vessel for additional synthesis. The oligosaccharide and host cells transformed with a polynucleotide that expresses a glycosyltransferase are added to sugar-nucleotides produced by a microorganism culture as needed until the desired oligosaccharide has been synthesized. Once the desired oligosaccharide has been synthesized, it is removed from the reaction vessel and exposed to further processing such as centrifugation or decantation, ion exchange chromatography, tangential flow, filtration, reverse osmosis or spray drying and lyophilization to obtain a pure oligosaccharide.

The process of the present invention can be used to synthesize any oligosaccharide. For example, the process of the present invention can be used to synthesize oligosaccharides such as Lacto-N-neoTetraose (LNnT), lacto-N-fucopentaose (LNF-V), 2'-fucosyllactose (2'-FL), 3-fucosyllactose (3-FL), lacto-N-fucopentaose III (LNF-III), lacto-N-fucopentaose II (LNF-II), difucosyllactose (DFL), lacto-N-fucopentaose (LNF-I) and Lacto-N-Tetraose (LNT).

The oligosaccharides produced, according to the process of this invention, find use in an exceedingly wide variety of applications and may be used in the same manner as saccharide compositions available from known sources. The present invention provides pharmaceuticals nutritional compositions and other oligosaccharide containing compositions prepared in accordance with the present invention.

The following examples illustrate preferred embodiments of the present invention and are not limiting of the claims and specification in any way.

Example 1: Production of the Oligosaccharide LNnT in a Single Reaction Vessel UDP-GlcNAc Yeast Production System

Dried yeast cells (*S. cerevisiae*) are fed 20 mM glucosamine, 170 mM KH_2PO_4, 5 mM $MgSO_4$, 70 mM fructose and 20 mM UMP. This yeast culture is incubated at room temperature. The yeast culture is monitored for the formation of

the sugar-nucleotide UDP-GlcNAc. When the amount of UDP-GlcNAc produced is approximately 6.0 mM, the sugar-nucleotide yeast production system is added to a reaction vessel.

Trisaccharide - LNT-2 (GlcNAc 1-3Gal 1-4Glc) Synthesis

E. coli cells expressing GlcNAc transferase (100 Units/Liter of Reaction) are homogenized, and, without any further purification, are added to the reaction vessel containing the UDP-GlcNAc production system. The GlcNAc transferase and UDP-GlcNAc yeast production system are incubated at room temperature until the residual UDP GlcNAc production is approximately 1.0 mM. 30 mM lactose is added to the reaction vessel as a substrate to produce material containing the trisaccharide intermediate LNT-2 (GlcNAcB1-3Gal 1-4Glc). The amount of LNT-2 containing material produced should be greater than 6 mM. *E. coli* whole cell homogenates may be substituted with *E. coli* intact cells +/- Triton X-100.

LNnT (Gal 1-4GlcNAc 1-3Gal 1-4Glc) Synthesis

E. coli cells expressing Gal transferase (350 Units/Liter of Reaction) and fortuitously an epimerase are homogenized and added to the reaction vessel without any purification. The Gal transferase, the LNT-2 containing material and the UDP-Glc/yeast solution are incubated at room temperature until the residual production of the GlcNAc 1-3Gal 1-4Glc is approximately 0.1 mM. The required UDP-Glc is produced by feeding dried yeast cells (*Candida famata*) 200-400 mM glucose (Glc), 180 mM KH_2PO_4, 12 mM $MgSO_4$ and 30-100 mM UMP. The yeast is incubated at room temperature for 10 - 48 hours and monitored for the production of UDP-Glc. When the amount of UDP-Glc produced exceeds 6 mM, the UDP-Glc yeast mixture is added to the reaction vessel to produce Lacto-N-neoTetraose (LNnT). The amount of LNnT containing material produced should be greater than 6 mM. The LNnT containing material is then removed from the reaction vessel and moved downstream for further processing such as by centrifugation or decantation, ion exchange chromatography, tangential flow filtration, reverse osmosis, spraying drying or lyophilization to obtain pure LNnT.

Example 2: Production of LNnT in a Single Reaction Vessel Using a Two Yeast System LNT-2 (GlcNAc 1-3Gal 1-4Glc) Synthesis

All of the reagents necesary for the synthesis of the intermediate LNT-2 are added simultaneously to one reaction vessel. More specifically, a mixture of dried *Candida famata* and *S. cerevisiae* are fed 20 mM glucosamine, 20 mM UMP, 170 mM KH_2PO_4, 5 mM $MgSO_4$, 70-175 mM fructose to produce the sugar-nucleotide UDP-GlcNAc. At the same time, 30 mM lactose and homogenized, unpurified *E. coli* cells expressing GlcNAc transferase (100 Units/Liter of Reaction Mix) are added. The entire reaction mixture is incubated at room temperature until the levels of LNT-2 containing material produced are greater than 6.0 mM.

LNnT (Gal 1-4GlcNAc 1-3Gal 1-4Glc) Synthesis

Additional homogenized, unpurified *E. coli* cells expressing Gal transferase (350 units/Liter of Reaction) and an epimerase are added to the reaction vessel. More specifically, dried *Candida famata* are fed 200-400 mM glucose, 180 mM KH_2PO_4, 12 mM $MgSO_4$ and 20-100 mM UMP. The yeast is incubated at room temperature until the amount of UDP-Glc produced exceeds 6mM. The sugar-nucleotide yeast producing system is then added to the reaction vessel containing the LNT-2 containing material and the *E. coli* cells expressing Gal transferase to produce LNnT containing material. The LNnT containing material is then removed from the reaction vessel and moved downstream for further processing such as by centrifugation or decantation, ion exchange chromatography, tangential flow, filtration, reverse osmosis, spraying drying or lyophilization to obtain pure LNnT.

Example 3: Production of LNnT In a Single Reaction Vessel Using a Two Yeast System by the Simultaneous Addition of Reagents

All of the reagents necessary for the synthesis of LNnT are added simultaneously to one reaction vessel. More specifically, a mixture of dried Baker's yeast (*S. cerevisiae*) and *Candida famata* are fed 30 mM glucosamine, 40 mM UMP, 170 mM KH_2PO_4, 12 mM $MgSO_4$ and 200 mM maltose to produce the sugar-nucleotides UDP-GlcNAc and UDP-Glc. At the same time, 30 mM lactose,

homogenized, unpurified *E. coli* cells expressing GlcNAc transferase (1900 U/L reaction) and homogenized, unpurified *E. coli* cells expressing Gal transferase (1000-2000 U/L reaction) are added to the same reaction vessel. The entire reaction mixture is incubated at room temperature and is allowed to proceed until the level of LNnT containing material is greater than 3 mM. The LNnT containing material is then removed from the reaction vessel and moved downstream for further processing such as by centrifugation or decantation, ion exchange chromatography, tangential flow, filtration, reverse osmosis, spraying drying or lyophilization to obtain pure LNnT.

Example 4: Production of the Oligosaccharide LNnT in a Single Reaction Vessel Using a Two Yeast System by the Exogenous Addition of LNT-2.

All of the reagents necessary for the synthesis of LNnT are added simultaneously to one reaction vessel. More specifically a mixture of dried Baker's yeast and *Candida famata* are fed 30 mM glucosamine, 40 mM UMP, 170 mM KH_2PO_4, 12 mM $MgSO_4$ and 200 mM maltose to produce the sugar-nucleotide UDP-GlcNAc. At the same time, 30 mM lactose, 30 mM LNT-2, homogenized, unpurified *E. coli* cells expressing GlcNAc transferase (1900 Units/Liter of Reaction Mix) and homogenized, unpurified *E. coli* cells expressing Gal transferase (1000 Units/Liter of Reaction mix) are added to the reaction vessel. The entire reaction mixture is refrigerated or incubated at room temperature and is allowed to proceed until the levels of LNnT containing material reach at least 15 mM. The LNnT containing material is then removed from the reaction vessel and moved downstream for further processing such as by centrifugation or decantation, ion exchange chromatography, tangential flow, filtration, reverse osmosis or spraying drying and lyophilization to obtain pure LNnT.

Example 5: Production of LNnT in a Single Reaction Vessel using a Single Yeast Production System

All of the reagents necessary for the synthesis of LNnT are added simultaneously to one reaction vessel. More specifically, dried *Candida famata* are

fed 30 mM glucosamine, 40 mM UMP, 170 mM KH_2PO_4, 12 mM $MgSO_4$ and 200 mM maltose to produce the sugar-nucleotides UDP-GlcNAc and UDP-Glc. At the same time, 30 mM lactose, intact *E. coli* cells expressing GlcNAc transferase (1900 Units/Liter of Reaction Mix) and intact *E. coli* cells expressing Gal transferase (2000 Units/Liter of Reaction mix) are also added to the reaction vessel. The entire reaction mixture is incubated at room temperature and is allowed to proceed until the levels of LNnT containing material reach at least 18 mM. The LNnT containing material is then removed from the reaction vessel and moved downstream for further processing such as by centrifugation or decantation, ion exchange chromatography, tangential flow, filtration, reverse osmosis, spraying drying or lyophilization to obtain pure LNnT.

Example 6: Production of LNnT in a Single Reaction Vessel using a Single Yeast Production System and Orotate

All of the reagents necessary for the synthesis of LNnT are added simultaneously to one reaction vessel. More specifically, dried *Candida famata* are fed 30 mM glucosamine, 40 mM orotate, 170 mM KH_2PO_4, 12 mM $MgSO_4$ and 200 mM glucose to produce the sugar-nucleotides UDP-GlcNAc and UDP-Glc. At the same time, 30 mM lactose, homogenized, unpurified *E. coli* cells expressing GlcNAc transferase (1900 Units/Liter of Reaction Mix) and homogenized, unpurified *E. coli* cells expressing Gal transferase (2000 Units/Liter of Reaction mix) are added to the reaction vessel. The entire reaction mixture is refrigerated or incubated at room temperature and is allowed to proceed until the levels of LNnT containing material reach at least 13 mM. The LNnT containing material is then removed from the reaction vessel and moved downstream for further processing such as by centrifugation or decantation, ion exchange chromatography, tangential flow, filtration, reverse osmosis or spraying drying and lyophilization to obtain pure LNnT.

Example 7: Synthesis of LNnT in a Single Reaction Vessel Using Single Yeast Production System and LNT-2

A method that can be used for synthesizing LNnT using a single yeast production system and LNT-2 will now be described. All of the reagents necessary for the synthesis of LNnT can be added simultaneously to one reaction vessel. More specifically, dried *Candida famata* is fed 30 mM glucosamine, 40 mM UMP, 170 mM KH_2PO_4, 12 mM $MgSO_4$ and 200 mM maltose to produce the sugar-nucleotides UDP-GlcNAc and UDP-Glc. At the same time, 30 mM lactose, 30 mM LNT-2, homogenized, unpurified *E. coli* cells expressing GlcNAc transferase (1900 Units/Liter of Reaction Mix) and homogenized, unpurified *E. coli* cells expressing Gal transferase (2000 Units/Liter of Reaction mix) are added to the reaction vessel. The entire reaction mixture is incubated at room temperature and is allowed to proceed until the levels of LNnT containing material reach at least 18 mM. The LNnT containing material is then removed from the reaction vessel and moved downstream for further processing such as by centrifugation or decantation, ion exchange chromatography, tangential flow, filtration, reverse osmosis or spraying drying and lyophilization to obtain pure LNnT.

Example 8: Production of the Oligosaccharide LNnT In Two Reaction Vessels Using a Two Yeast System

UDP-GlcNAc Production System

Dried *Baker's yeast* are fed 20 mM glucosamine, 170 mM KH_2PO_4, 5 mM $MgSO_4$ and 70 mM fructose. 20 mM UMP is added to the yeast. The yeast are incubated at room temperature and monitored for the formation of the sugar-nucleotide UDP-GlcNAc. The yeast are incubated at room temperature until the amount of UDP-GlcNAc produced is approximately 6.0 mM. The sugar-nucleotide yeast producing system is added to a first reaction vessel.

Trisaccharide - LNT-2 (GlcNAc 1-3Gal 1-4Glc) Synthesis

E. coli cells expressing GlcNAc transferase (350 units/Liter of Reaction Mix) are homogenized and, without any further purification, are added to the reaction vessel containing the UDP-GlcNAc yeast production system. The GlcNAc transferase and UDP-GlcNAc production system are incubated at room

temperature until the residual UDP-GlcNAc production is less than 1.0 mM. Lactose is also added to the reaction vessel as a substrate to produce material containing the trisaccharide intermediate LNT-2 (GlcNAc -3Gal 1-4Glc). The amount of LNT-2 containing material produced should be greater than 6 mM. The LNT-2 containing material is removed from the first reaction vessel and transported to a second reaction vessel.

LNnT (Gal 1-4GlcNAc 1-3Gal 1-4Glc) Synthesis

Additional homogenized and unpurified *E. coli* cells expressing Gal transferase (350 units/Liter of Reaction) are added to the second reaction vessel containing the LNT-2 containing material and a source of UDP-Glucose. This material is incubated at room temperature until the residual production of the GlcNAc 1-3Gal 1-4Glc is approximately 0.1 mM. The source of UDP-Glc is supplied by incubating *Candida famata*, 200-400 mM glucose, 180 mM KPO_4, 12 mM $MgSO_4$ and 20-100 mM UMP. The microorganisms are incubated at room temperature until the amount of UDP-Glc produced is greater than 6 mM. The sugar-nucleotide yeast producing system is then added to the second reaction vessel containing the trisaccharide GlcNAc 1-3Gal 1-4Glc and the Gal transferase to produce LNnT. The material containing the LNnT is then removed from the second reaction vessel and moved downstream for further processing such as by centrifugation or decantation, ion exchange chromatography, tangential flow, filtration, reverse osmosis or spraying drying and lyophilization to obtain pure LNnT.

Example 9: Production of the Oligosaccharide LNnT in a Single Reaction Vessel Using a Single Yeast System

Trisaccharide (LNT-2, GlcNAc 1-3Gal 1-4Glc) Synthesis in the Absence of Added Nucleotide

To dried *Candida famata* cells, 50 mM glucosamine, 200 mM KH_2PO_4, 12 mM $MgSO_4$, 200 mM maltose, 100 mM lactose and homogenized, unpurified *E. coli* cells expressing GlcNAc transferase (1200 Units/Liter reaction mix) are added.

The entire mixture is incubated at room temperature with aeration. After 24 hours incubation, the reaction is supplemented with an additional 40 mM glucosamine and 200 mM maltose. The mixture is further incubated until greater than 55 mM LNT-2 is generated.

LNnT (Gal 1-4GlcNAc 1-3Gal 1-4Glc) Synthesis

To the yeast/LNT-2 mix, air dried *Candida famata*, 400 mM glucose (or galactose), 180 mM KH_2PO_4, 12 mM $MgSO_4$ and homogenized, unpurified *E. coli* cells expressing Gal transferase (1000 Units/Liter reaction mix) are added. The reaction is incubated at room temperature with aeration until at least 50 mM LNnT is produced. The LNnT containing material is then removed from the reaction vessel and moved downstream for further processing such as by centrifugation or decantation, ion exchange chromatography, tangential flow filtration, reverse osmosis, spray drying or lyophilization to obtain pure LNnT.

Example 10: Production of the Oligosaccharide LNnT in Two Reaction Vessels Using a Single Yeast System

Trisaccharide (LNT-2, GlcNAc 1-3Gal 1-4Glc) Synthesis in the Absence of Added Nucleotide

To dried *Candida famata* cells, 50 mM glucosamine, 200 mM KH_2PO_4, 12 mM $MgSO_4$, 200 mM maltose, 100 mM lactose and homogenized, unpurified *E. coli* cells expressing GlcNAc transferase (1200 Units/Liter reaction mix) are added. The entire mixture is incubated at room temperature with aeration. After 24 hours incubation, the reaction is supplemented with an additional 40 mM glucosamine and 200 mM maltose. The mixture is further incubated until greater than 55 mM LNT-2 is generated. The LNT-2 is semi-purified by a combination of methods including centrifugation, ion exchange chromatography, tangential flow filtration and reverse osmosis. This LNT-2 material is transferred to a second reactor.

LNnT (Gal 1-4GlcNAc 1-3Gal 1-4Glc) Synthesis

In the second reactor, air dried *Candida famata*, 400 mM glucose (or galactose), 180 mM KH_2PO_4, 12 mM $MgSO_4$, 100 mM UMP, the LNT-2 material and homogenized, unpurified *E. coli* cells expressing Gal transferase (1000 Units/Liter reaction mix) are added. The reaction is incubated at room temperature with aeration until at least 50 mM LNnT is produced. The LNnT containing material is then removed from the reaction vessel and moved downstream for further processing such as by centrifugation or decantation, ion exchange chromatography, tangential flow filtration, reverse osmosis, spray drying or lyophilization to obtain pure LNnT.

Example 11: Production of the Oligosaccharide LNnT in a Single Reaction Vessel Using a Single Yeast System in the Absence of Added Nucleotide.

All of the reagents necessary for the synthesis of LNnT are added simultaneously to one reaction vessel. More specifically, dried *Candida famata* are fed 50 mM glucosamine, 200 mM KH_2PO_4, 12 mM $MgSO_4$, 100 mM maltose to produce the sugar-nucleotides UDP-GlcNAc and UDP-Glc. At the same time, 100 mM lactose and homogenized, unpurified *E. coli* cells expressing GlcNAc Transferase (1500 Units/Liter reaction mix) are added to the reaction vessel. The entire mixture is incubated at room temperature with aeration. After 18 hours, the reaction is supplemented with an additional 50 mM glucosamine and 100 mM maltose. After 23 hours, 200 mM galactose and homogenized, unpurified *E. coli* cells expressing Gal transferase (1000 Units/Liter reaction mix) are added to the reaction vessel. The reaction is allowed to proceed until the levels of LNnT reach at least 40 mM (approximately 46 hours). The LNnT containing material is then removed from the reaction vessel and moved downstream for further processing such as by centrifugation or decantation, ion exchange chromatography, tangential flow filtration, reverse osmosis, spray drying or lyophilization to obtain pure LNnT.

Example 12: Production of the Oligosaccharide Lacto-N-fucopentaose III (LNF-III) in a Single Reaction Vessel by the Addition of LNnT.

A method that can be used for synthesizing the oligosaccharide lacto-N-fucopentaose (LNF-III) will now be described. More specifically, dried *Torulopsis candida* are fed 30 mM fucose, 40 mM GMP and 30 mM fructose to produce the sugar-nucleotides GMP-fucose. At the same time, 20 mM LNnT-2 and homogenized, unpurified *E. coli* cells expressing -1,3 fucosyltransferase (1900 Units/Liter of Reaction Mix) are homogenized and added without any purification to the reaction vessel. The entire reaction mixture is incubated at room temperature and is allowed to proceed until the levels of LNF-III containing material reach at least 5 mM. The LNF-III containing material is removed from the reaction vessel and moved downstream for further processing such as centrifugation or decantation, ion exchange chromatography, tangential flow, filtration, reverse osmosis, spraying drying or lyophilization to obtain pure LNF-III.

WHAT IS CLAIMED IS:

1. A process of synthesizing an oligosaccharide comprising the steps of:

a) adding an acceptor moiety and a catalytic amount of a glycosyltransferase to a microorganism culture that produces a sugar-nucleotide having a sugar unit to produce a mixture wherein the glycosyltransferase catalyzes the transfer of the sugar unit from the sugar-nucleotide to the acceptor molecule in the mixture; and

b) maintaining the mixture under conditions and for a period of time sufficient for oligosaccharide formation.

2. The process of claim 1 wherein the acceptor moiety is a monosaccharide.

3. The process of claim 1 wherein the acceptor moiety is a di, tri- or oligosaccharide.

4. The process of claim 1 wherein the glycosyltransferase is produced by host cells transformed with a polynucleotide that encodes the glycosyltransferase.

5. The process of claim 4 wherein the transformed cells are added to the microorganism culture.

6. The process of claim 4 wherein the glycosyltransferase is contained in a culture medium of the host cells.

7. The process of claim 1 wherein the microorganism is from the genus *Saccharomyces, Zygosaccharomyces, Torulopsis, Candida, Cryopcoccus, Brettanomyces, Mucor, Hansenula*, or *Debaryomyces*.

8. The process of claim 7 wherein the microorganism is *Saccharomyces cerevisiae, Candida famata,* or *Zygosaccharomyces rouxii.*

9. The process of claim 1 wherein the microorganism culture further comprises an epimerase.

10. The process of claim 1 wherein the saccharide nucleotides are UDP-glucose, UDP-N-acetylgalactosamine, UDP-Galactose, UDP-N-acetylgalactosamine, GDP-Mannose, GDP-fucose or CMP-N-acetylneuraminic acid.

11. The process of claim 1 wherein the mixture is maintained for about 4 to about 48 hours, at a temperature from about 4 C to about 35 C and at a pH from about 4 to about 9.

12. The process of claim 1 wherein the acceptor molecule and the glycosyltransferase are added at the same time.

13. The process of claim 1 wherein the acceptor moiety is added before the glycosyltransferase.

14. A process of synthesizing an oligosaccharide comprising the steps of:

a) adding an acceptor moiety and host cells transformed with a polynucleotide that encodes a catalytic amount of a glycosyltransferase to sugar-nucleotides having a sugar unit to produce a mixture wherein the glycosyltransferase catalyzes the transfer of the sugar unit of the sugar-nucleotide to the acceptor moiety in the mixture; and

b) maintaining the mixture under conditions and for a period of time sufficient for oligosaccharide formation.

15. The process of claim 14 wherein the acceptor moiety is a monosaccharide.

16. The process of claim 14 wherein the acceptor moiety is a di, tri- or oligosaccharide.

17. The process of claim 14 wherein the saccharide nucleotides are UDP-glucose, UDP-N-acetylgalactosamine, UDP-Galactose, UDP-N-acetylgalactosamine, GDP-Mannose, GDP-fucose or CMP-N-acetylneuraminic acid.

18. The process of claim 14 wherein the sugar-nucleotide is contained in a culture of microorganisms.

19. The process of claim 18 wherein the microorganism is from the genus *Saccharomyces, Zygosaccharomyces, Torulopsis, Candida, Cryopcoccus, Brettanomyces, Mucor, Hansenula,* or *Debaryomyces*.

20. The process of claim 14 wherein the mixture is maintained from about 4 to about 48 hours, at a temperature from about 4 C to about 35 C and at a pH from about 4 to about 9.

21. The process of claim 14 wherein the acceptor molecule and the host cells are added at the same time to the sugar-nucleotide.

22. The process of claim 14 wherein the acceptor moiety is added to the sugar-nucleotide before the host cells.

INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12P19/18

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO 95 02683 A (NEOSE PHARMACEUTICALS) 26 January 1995 see page 6, paragraph 4 - page 9, paragraph 1 see page 16, paragraph 6 - page 18, paragraph 1; example 1 ---	1-8, 10-22 9
Y	US 5 516 665 A (WONG) 14 May 1996 see column 2, line 38 - column 3, line 7 --- -/--	9

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

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"E" earlier document but published on or after the international filing date

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 91 16449 A (THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA) 31 October 1991 cited in the application see page 8, line 13 - line 21 see page 9, line 24 - page 10, line 17 see page 12, line 17 - page 15, line 14 see page 17, line 2 - line 26 ---	1-22
A	WO 96 10086 A (THE ROCKEFELLER UNIVERSITY) 4 April 1996 cited in the application see page 27, line 7 - page 28, line 22 see page 30, line 11 - page 31, line 25 see page 33, line 1 - line 7 -----	1-22

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